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(54) Title: HOMOGENEOUS ERYTHROPOIETIN

(57) Abstract

Homogeneous erythropoietin for the first time characterized by an amino acid sequence 1-166 substantially as sh wn in table 1, molecular weight of about 34,000 daltons, movement as a single peak on reverse phase HPLC, and specific activity of at least 120,000 IU.

# HOMOGENEOUS ERYTHROPOIETIN

Erythrocytes (also called red blood cells) serve a critical role in carrying oxygen to mammalian tissues. They are produced by the maturation and differentiation of erythroblasts in bone marrow. Erythropoietin (also referred to as EPO) is a glycoprotein which naturally stimulates erythrocyte formation in mammals.

In certain clinical states, e.g. various anemias, the level of erythrocytes is undesirably low. Exogenously administered EPO has promise as a therapeutic agent for the clinical treatment of such conditions. For therapeutic use, it is highly desirable that the EPO be homogeneous. Unfortunately, exogenous EPO is not in practical use due to its low availability and heterogeneity.

The preparation heretofore of erythropoietin-related products has generally been via the concentration and purification of urine from patients exhibiting high EPO levels. See, for example, U.S. Patent Nos. 3,865,801; 4,303,650 and 4,397,840. In particular, EPO has been purified from the urine of patients with aplastic anemia by the method described by Miyake et al., J. Biol. Chem., 252:5558 (1977), the disclosure of which is hereby incorporated by reference. It had been thought that EPO purified by this method was homogeneous because it moved as a single band during electrophoresis.

We have now discovered that this seemingly homogeneous EPO composition produced by the method of Miyake et al. is composed of several polypeptide components ranging from 30,000 to 70,000 daltons.

The present invention provides for the first time a homogeneous EPO composition. We prepared such a composition by subjecting an erythropoietin solution which has been partially purified by the Miyake-type method to reverse phase high performance liquid chromatography and eluting the EPO protein. We thereby produced homogeneous EPO charact-

erized by (a) an amino acid sequence substantially as shown in Table 1, (b) movement as a single peak on reverse phase-HPLC, (c) a molecular weight of about 34,000 daltons on SDS PAGE, and (d) a specific activity of at least 120,000 IU per absorbance unit at 280 nanometers.

Brief Description of the Drawings and Table

FIG. 1 is an elution profile of an EPO composition treated by reverse phase high performance liquid chromatography in accord with the present invention illustrating absorbance of fractions at 280 nm versus time.

Table 1 is the amino acid sequence of a human EPO protein including its secretory leader sequence.

The availability of homogeneous EPO will permit the sequencing of its amino acid composition by techniques known in the art to permit the construction of probes with which to "fish out" the corresponding human genes and thus facilitate production of EPO by recombinant DNA techniques.

In fact, an operable recombinant DNA process for producing EPO which relies on sequence information obtained through the use of EPO of this invention is described in copending and commonly assigned United States patent applications Serial No. 688,622 filed January 3, 1985 and Serial No. 693,258 filed January 22, 1985.

The method of Miyake et al. comprises deactivating any proteolytic enzymes by treating the crude EPO preparations with phenol p-aminosalicylate. Such proteases can also be deactivated by other means such as by heating. The purification steps described by Miyake et al. include ethanol precipitation, DEAE-agarose fractionation, sulfopropyl-Sephadex chromatography, gel filtration and hydroxylapatite chromatography. The "purified" EPO compositions obtained thereby reportedly have a specific EPO activity of at least about 50,000, preferably at least about to 80,000 IU, per

absorbance unit at 280 nm. (The prior art thus considered "pure" EPO as having a specified activity of about 80,000 IU).

We found the "purified" Miyake EPO composition to be non-homogeneous. By further treating this composition by reverse phase high performance liquid chromatography (R-P HPLC) we obtained a homogeneous EPO having a molecular weight of about 34,000 daltons when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

A preferred reverse phase HPLC column for use in this invention is a column having four-carbon (butyl) chain groups attached thereto. A commercial example is the C-4 Vydac column available from The Nest Group, Southboro, MA. Other reverse columns with different carbon chain groups, such as eight-carbon or eighteen-carbon, may also be used. A preferred eluant consists of a 0 to 95% acetonitrile gradient in 0.01 to 1.0%, preferably 0.1% trifluoroacetic acid, over a period of about 100 minutes. Other eluants can of course also be used.

When purified in accordance with the present invention, EPO compositions having a specific activity of at least 120,000 IU per absorbance unit at 280 nm are obtained. In a preferred embodiment specific activities of at least 160,000 IU are obtained. An "absorbance unit," as used herein, is approximately 1 mg protein per ml.

The amino acid sequence of an EPO protein derived from a human source, including its secretory leader sequence is illustrated in Table I. The mature EPO protein begins with the "Ala" residue identified by the arabic number "1". The secretory leader sequence is the polypeptide sequence preceding the mature EPO protein beginning with th "MET" residue identified by the numeral "-27". This DNA sequence can be expressed in a cell capable of processing the EPO protein to eliminate the leader sequence and secrete the mature protein int the media.

In clinical uses, e.g., in the treatment of various anemias, the amount of EPO will, of course, depend upon the severity of the condition being treated, the route of administration chosen, and the specific activity of the EPO, and ultimately will be decided by the attending physician.

EPO may be administered by any route appropriate to the condition being treated. Preferably, the EPO is injected into the bloodstream.

The formulations of the present invention comprise an active EPO protein, as above described, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

Formulations suitable for parenteral administration conveniently comprise a sterile parenterally acceptable vehicle, e.g., water containing a therapeutically effective amount of EPO. Solutions are preferably isotonic.

The following example illustrates the preparation of the product of the present invention.

## EXAMPLE

Crude erythropoietin preparations, derived from patients with aplastic anemia, were concentrated by dialysis. Proteolytic enzymes were deactivated by heat treatment at 80°C for 5 minutes. The crude preparation concentrates were then purified by the above method described by Miyake et al.

A. Ethanol Precipitation

Batches containing about 100,000 IU of EPO activity at concentrations of about 50 to 100 IU per absorbance unit at 280 nm were diluted to 50 ml with phosphate buffered solution (PBS) at 4°C. 12.5 ml of 10 M LiCl were added. Absolute

thanol (62.5 ml) at 4° was added slowly with stirring, which was continued for 30 min. after the addition was complete. After the flocculent precipitate had been allowed to settle for 10 min. it was removed by centrifugation at 21,000 x g for 10 min at -15°. The pellet was washed three times with 10 ml of 50% ethanol, 1 m LiCl and the supernatants were pooled. The washed precipitate was dissolved in 20 ml of PBS, yielding a turbid solution (50% precipitate.)

Sixty-seven milliliters of absolute ethanol were added slowly to the combined supernatants: stirring was continued for 30 min. and settling for 15 min. The precipitate was collected as before and washed twice with 10 ml of 65% ethanol, 0.7 M LiCl and the supernatants were pooled. The washed precipitate was dissolved in 20 ml of PBS (65% precipitate).

To the pooled supernatants, 96 ml of ethanol were added slowly and stirring was continued for 30 min. after which the precipitate was allowed to settle for 14 hrs. at 4°. The precipitate was washed twice with 10 ml of 75% ethanol, 0.5 M LiCl, the supernatants were pooled, and the precipitate was dissolved in 20 ml of PBS (75% precipitate).

The combined supernatant was brought to 90% ethanol by addition of 540 ml of absolute alcohol, stirred for 30 min. and stored at -20°for 48 hrs. before the precipitate was collected, dissolved in 50 ml of cold water and immediately frozen.

B. DEAE - Agarose Fractionation

The solution, in water, of a 90% ethanol precipitate was concentrated to about 5 ml of an Amicon UM-10 ultrafilter, then brought to 25 ml with 0.01 M Tris, pH 7.0, and a 50-ul aliquot was removed. The DEAE-agarose, 100 to 200 mesh, was degassed under reduced pressure, suspended in 0.01 M Tris, pH 7.0, and packed int a column 9.2 x 2.5 cm in diam ter (bed volume, 45 ml). The gel was wash d with 1.5 liters of 0.01 M Tris, pH 6.9; the ratio of absorbance

units added t bed volume (ml) was 6.65. The sample was added to the column over a peri d f 40 min. and 150-dr p fractions were c ll cted. The column was washed with 211 ml of 0.01 M Tris, pH 7, and then eluted with the following buffers: 366 ml of 0.01 M Tris, pH 7.0; 5mM $CaCl_2$, 270 ml of 0.01 Tris, pH 7.0; 17 mM $CaCl_2$; 194 ml of 0.01 M Tris, pH 7.0;3 mM $CaCl_2$; and 65 ml of 0.1 M $CaCl_2$.

From this point on in the fractionation calcium was added to all buffers except those used with hydroxylapatite columns because there were inconsistent results and appreciable losses of activity when buffers without calcium were used. For the next step in purification, eluates from DEAE agarose columns were selected that had significant quantities of EPO activity.

C. Sulfopropyl-Sephadex Chromatography

The eluates (17 mM $CaCl_2$) from DEAE-agarose columns were desalted and concentrated on a UM-10 ultafilter and then dialyzed against 2 liters of 5 mM $CaCl_2$, pH 7.5 overnight. In the sample run described below, 30 ml of dialyzed solution were brought to pH 4.50 by dropwise addition of 0.1 M HCl: the small amount of precipitate formed was removed by centrifugation and washed with 5 ml of 5 mM $CaCl_2$, pH 4.5. The wash, pooled with the supernatant, was applied to a sulfopropyl-Sephadex column (15.0 x 2.5 cm in diameter, bed volume, 78.3 ml) which had been equilibrated with 5 mM $CaCl_2$, pH 4.50. The absorbance units to bed volumn (ml) ratio was 2.47. A low value for this ratio is preferred for optimal fractionation on sulfopropyl-Sephadex: for example, if the absorbance unit to bed volume ratio was greater than 10, almost all of the activity was found in the effluent fraction. The following buffers were used in developing the column. Input was: 5 mM calcium acetate, pH 4.50, specific conductivity = 1.075 umh $cm^{-1}$. Eluting buffers were: 7.5 mM calcium acetate, pH 4.70, specific conductivity = 2,100 umho $cm^{-1}$: 15 mM calcium acetate, pH

5.25, specific conductivity = 2,100 umho $cm^{-1}$: 15 mM calcium acetate, 0.01 m Tris, pH 7.24, specific c nductivity = 11,500 umh $cm^{-1}$. The column was run at 0.4 ml/min. at 4°, and 200-drop fractions were collected. After a reading was taken at 280 nm and the appropriate pools were made, the solutions were neutralized (within 1 hr. after elution) and aliquots were removed for assay and stored at -20°.

D. Gel Filtration

The 12.5 and 15 mM calcium acetate eluates from the sulfopropyl-Sephadex column separations were run in tw separate batches on the same gel column. The pools were concentrated on Amicon UM-2 ultrafilters to about 5 ml and equilibrated with 10 mM $CaCl_2$, 10 mM Tris, pH 6.87, before application to the column. The Sephadex G-100 gel was degassed under reduced pressure and equilibrated with the same buffer before the column was poured. The column (100 x 2.5 cm diameter) was calibrated with markers of known molecular size before being used for the erythropoietin fractions. The void volume was 135 ml; bovine serum albumin monomer eluted at 224 ml. ovalbumin at 258 ml, and cytochrome at 368 ml. The sample was added to the bottom of the column, as was the buffer which was passed through the column at 21 to 22 ml by means of Mariotte bottle with a 42 cm hydrostatic head. Each fraction collected was 4.1 ml (120 drop), and pools were made. The pools were concentrated by ultrafiltration and aliquots were assayed.

E. Hydroxylapatite Chromatography

Hydroxylapatite was packed under unit gravity into a column (6.1 x 1.5 cum diameter) and washed with 500 ml of water and then with 400 ml of 0.5 mM phosphate buffer, pH 7.1, conductivity = 69 umho $cm^{-1}$ (Buffer I), by use of a peristaltic pump which maintained the flow at 0.3 ml/min. After the buffer wash, the length of the column was 3.4 cm and the bed volume was 6.0 ml. the input sample was concentrated and desalted on an Amicon DM-5 ultrafilter by adding

wat r t the concentrate and the wash of the filt r was centrifuged at 6,000 x g for 20 min. at 4°. The small ins luble pellet was washed once with 0.5 mM phosphate, pH 7.1, and the wash was added to the supernatant. An aliquot for assay was removed and the remainder (22 ml) was added to the column. The ratio of absorbance units added to bed volume (ml) was 1.82. The input buffer was pumped through the column until the effluent A was less than 0.005 (149 ml) and the following elution schedule was carried out: Buffer II, 1 mM phosphate (pH 7.1, specific conductivity 131 = umho cm $^{-1}$, 150 ml (Fraction II)); Buffer III, 2 mM phosphate (pH 6.9, specific conductivity = 270 umho cm$^{-1}$, 220 ml (fractions IIIA and IIIB)); Buffer IV, 3 mM phosphate (pH 6.9, specific conductivity = 402 umho cm$^{-1}$, 84 ml (Fraction IV)); Buffer V, 0.1 M phosphate (pH 6.8, specific conductivity = 9.6 umho cm$^{-1}$, 134 ml (Fraction V)).

Fractions containing EPO were concentrated by means of Amicon DM-5 ultrafilter, an aliquot assayed and the concentrate stored frozen. The assay indicated a specific EPO activity of 83,000 IU per absorbance unit at 280 nm.

F. Reverse Phase HPLC

When analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) according to Laemmli, U.K., Nature, 15:Vol. 22, No. 259 pp. 680-685 (1970) the material from the hydroxylapatite column revealed several polypeptide components ranging from approximately 70,000 MW to 30,000 MW with the major component at about 34,000 MW. This preparation of EPO which was obtained in volumes of up to 10 ml in 10 mM phosphate buffer pH 7.0 was subjected to R-P HPLC as described below.

The EPO preparation was concentrated 10-fold by partial lyophilization. Approximately 200 microliters of this c ncentrated mat rial was injected onto a R-P HPLC c lumn, the type with four-carbon groups attached (a commercial example is a C-4 Vydec), (25 x 0.45 cm, the S para-

ti ns Gr up) and fractionated by a R-P HPLC using the gradient conditions describ d in Table 2.

TABLE 2

Pump A 0.1% Trifluoracetic Acid (TFA) in water
Pump B 0.1% TFA in 95% Acetonitrile; 5% $H_2O$

| Gradient Time (Min.) | % B | Duration |
|---|---|---|
| 0 | 0 | 2 |
| 2 | 25 | 3 |
| 5 | 100 | 75 |
| 90 | 0 | 3 |

Flow 1 ml/min.

EPO is quantified by either the 3H-thymidine assay (Krystal, Exp. Hematol. 11:649-60 (1983)) or CFU-E assay (Bersch et al., In vitro Aspects of Erythropoiesis, M. J. Murphy (Ed.), New York: Springer-Verloz (1978)).

Protein peaks were detected by UV absorption at 280 nm. A typical elution profile of this fractionation process is shown in Fig. 1. The homogenous nature of EPO was confirmed by SDS PAGE and N-terminal amino acid sequence analysis of the various peaks observed after R-P HPLC. The peak that is underlined in Fig. 1 elutes coincidentally with a reading of 53% on the gradient maker (Beckman Instruments, model 421). This material runs as a single band of about 34,000 MW using SDS PAGE and yields a single amino terminal sequence of: Ala, Pro, Pro, Arg, Leu, Ile, Cys - as has been previously reported for human EPO. Only this R-P HPLC fraction of about 34,000 MW showed any significant biological activity in vitro. The EPO protein eluted by R-PHPLC is about twice as pure as the material eluted from the hydroxylapatite column (STEP E).

WHAT IS CLAIMED IS:

1. Homogeneous erythropoietin, characterized by:
   a) an amino acid sequence substantially as shown from 1-166 in Table 1;
   b) movement as a single peak on a reverse phase-HPLC;
   c) a molecular weight of about 34,000 dalton on SDS PAGE; and
   d) a specific activity of at least 120,000 IU per absorbance at 280 nm.

2. Homogeneous erythropoietin according to claim 1 wherein the specific activity is at least 160,000 IU.

3. A pharmaceutical composition for the treatment of anemia comprising a therapeutically acceptable amount of erythropoietin of claim 1 in a parenterally acceptable vehicle.

4. A pharmaceutical composition for the treatment of anemia comprising a therapeutically acceptable amount of erythropoietin of claim 2 in a parenterally acceptable vehicle.

**AMENDED CLAIMS**

[received by the International Bureau on 15 April 1986 (15.04.86); original claims 1-4 unchanged; new claims 5-8 added (2 pages)]

1. Homogeneous erythropoietin, characterized by:
   a) an amino acid sequence substantially as shown from 1-166 in Table 1;
   b) movement as a single peak on a reverse phase-HPLC;
   c) a molecular weight of about 34,000 dalton on SDS PAGE; and
   d) a specific activity of at least 120,000 IU per absorbance at 280 nm.

2. Homogeneous erythropoietin according to claim 1 wherein the specific activity is at least 160,000 IU.

3. A pharmaceutical composition for the treatment of anemia comprising a therapeutically acceptable amount of erythropoietin of claim 1 in a parenterally acceptable vehicle.

4. A pharmaceutical composition for the treatment of anemia comprising a therapeutically acceptable amount of erythropoietin of claim 2 in a parenterally acceptable vehicle.

5.(new) Homogeneous erythropoietin, characterized by:
   a) movement as a single peak on a reverse phase-HPLC;
   b) a molecular weight of about 34,000 dalton on SDS PAGE; and
   c) a specific activity of at least 120,000 IU per absorbance at 280 nm.

6.(new) Homogeneous erythropoietin according to claim 5 wherein the specific activity is at least 160,000 IU.

7.(new) A pharmaceutical composition for the treatment of anemia c mprising a therapeutically acceptable amount of erythropoietin of claim 5 in a parenterally acceptable vehicle.

8.(new) A pharmaceutical composition for the treatment of anemia comprising a therapeutically acceptable amount of erythropoietin of claim 6 in a parenterally acceptable vehicle.

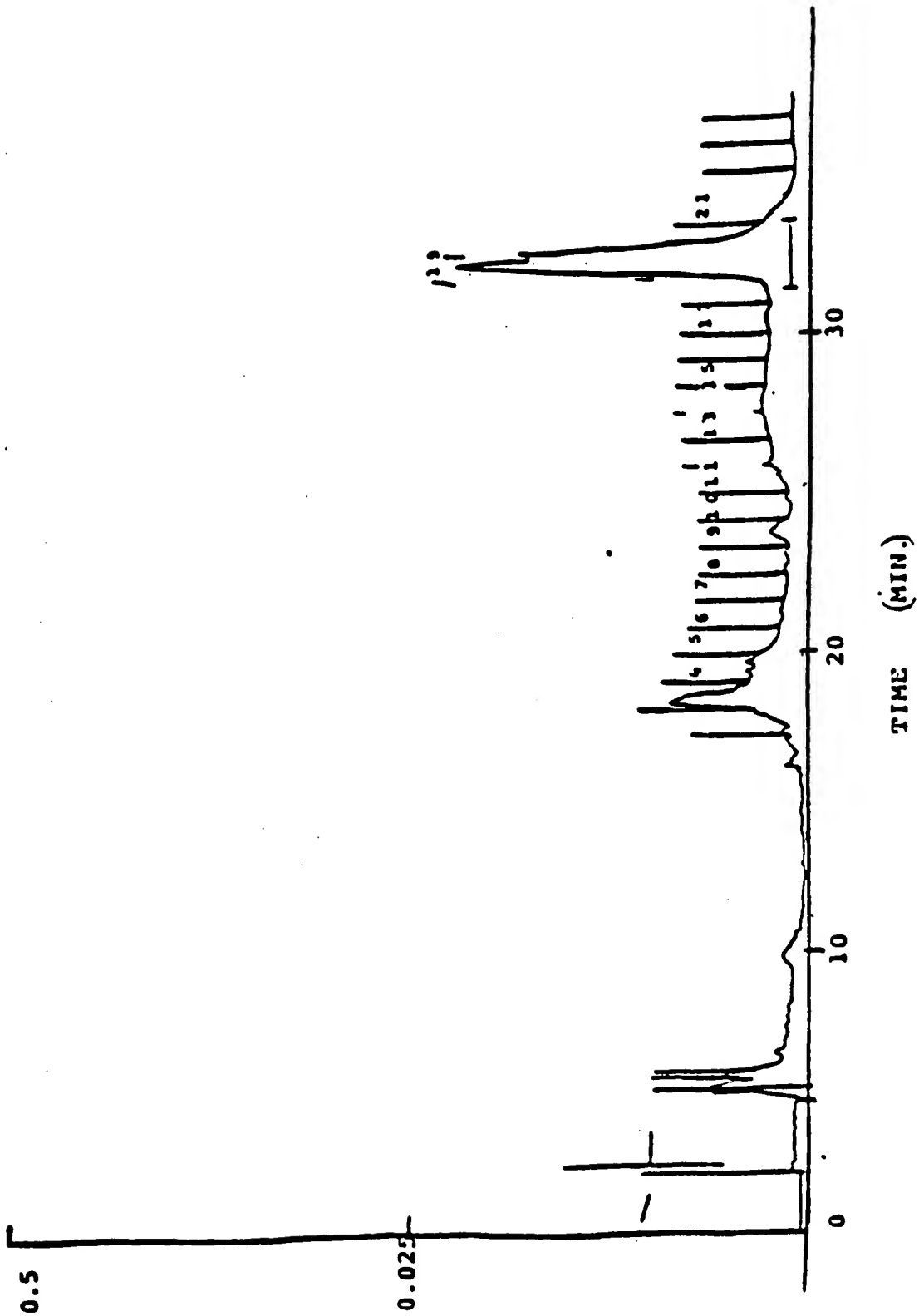


FIG.1

TABLE 1

| | | | | | | | | | | | | | -27 MET | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ALA | TRP | LEU | TRP | LEU | LEU | LEU | SER | LEU | LEU | SER | LEU | PRO | LEU | GLY | LEU | PRO | VAL | LEU | GLY |
| 1 Ala | Pr | Pro | Arg | Leu | Ile | Cys SH | Asp | Ser | 10 Arg | Val | Leu | Glu | Arg | Tyr | Leu | Leu | Glu | Ala | 20 Lys |
| Gln | Ala | Glu | Asn | Ile | Thr | Thr | Gly | Cys SH | 30 Ala | Glu | His | Cys | Ser | Leu | Asn | Glu | Asn | Ile | 50 Thr |
| Val | Pr | Asp | Thr | Lys | Val | Asn | Phe | Tyr | 50 Ala | Trp | Lys | Arg | Met | Glu | Val | Gly | Gln | Gln | 60 Ala |

TABLE 1 (CONTI'D)

| | | | | | | | | | 70 | | | | | | | | | 80 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Val | Glu | Val | Trp | Gln | Gly | Leu | Ala | Leu | Leu | Ser | Glu | Ala | Val | Leu | Arg | Gly | Gln | Ala | Leu |
| Leu | Val | Asn | Ser | Ser | Gln | Pro | Trp | Glu | 90 Pro | Leu | Gln | Leu | His | Val | Asp | Lys | Ala | Val | 100 S |
| Gly | Leu | Arg | Ser | Leu | Thr | Thr | Leu | Leu | 110 Arg | Ala | Leu | Gly | Ala | Gln | Lys | Glu | Ala | Il | 120 Ser |
| Pro | Pr | Asp | Ala | Ala | Ser | Ala | Ala | Pro | 130 Leu | Arg | Thr | Ile | Thr | Ala | Asp | Thr | Ph | Arg | 140 Lys |
| Leu | Phe | Arg | Val | Tyr | Ser | Asn | Phe | Leu | 150 Arg | Gly | Lys | Leu | Lys | Leu | Tyr | Thr | Gly | Glu | 160 Ala |
| Cys SH | Arg | Thr | Gly | Asp | 166 Arg | | | | | | | | | | | | | | |

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US/02358

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.4 C07K 7/10, 13/00; A61K 37/24
U.S. CL. 260/112R, 112.5R; 424/99; 435/68; 514/8

## II. FIELDS SEARCHED

**Minimum Documentation Searched [4]**

| Classification System | Classification Symbols |
|---|---|
| U.S. | 260/112R, 112.5R; 424/99; 435/68; 514/8 |

**Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]**

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

| Category [15] | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| X | US, A, 4,254,095 PUBLISHED 03 MARCH 1981 FISHER ET AL | 1-4 |
| Y | US, A, 4,289,690 PUBLISHED 15 SEPTEMBER 1981 PESTKA ET AL | 1-4 |
| Y | US, A, 4,377,492 PUBLISHED 22 MARCH 1983 RIVIER | 1-4 |
| X | US, A, 4,377,513 PUBLISHED 22 MARCH 1983 SUGINOTO ET AL | 1-4 |
| X | US, A, 4,465,624 PUBLISHED 14 AUGUST 1984 OHIBA ET AL | 1-4 |
| A,E | US, A, 4,558,005 PUBLISHED 10 DECEMBER 1985 GOLDWASSER ET AL | 1-4 |
| A,E | US, A, 4,558,006 PUBLISHED 10 DECEMBER 1985 EGRIE | 1-4 |
| X | N, MIYAKE ET AL, J.Biol. Chem. 252, NO.15, (1977), 5558-5564. | 1-4 |
| Y | N, PARSONS ET AL, Endocrinology, 114, NO.6, (1984), 2223-2227. | 1-4 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 28 JANUARY 1986 | 21 FEB 1986 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [20]** |
| ISA/US | Howard E. Schain |